Indonesian Journal of Biotechnology, June (2001) 439 - 447

Use of GFP (Green Fluorescent Protein) for monitoring annexin 1 function in phagocytosis

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Abstract

Annexin 1 belongs to a family of proteins that bind negatively charged phospholipids and membranes in a calcium dependent manner. Most of the proposed functions concern processes involving membrane fusion and/or fission such as that involved in phagocytosis. Latex beads were used in this study. cDNA of wild-type annexin 1 was expressed by transfection as a fusioned protein to GFP (green flourescent protein) at its N- terminal end. It turned out that, despite the regulatory importance of the N- terminal domain, the overexpression and the fusion of annexin 1 to GFP did not significantly alter the phagosome colocalization and this protein remained associated with the phagosomal protein complex during the maturation process. As GFP is becoming an important reporter molecule for monitoring gene expresion and protein localization in vivo, in situ and in real time, the significance of this finding on the physiological function of annexin 1 will be discussed.

Keywords: *annexin 1 – GFP – J-774A 1 – phagocytosis – protein expression.*

Introduction

Annexin 1 belongs to a family of ubiquitous proteins that bind negatively charged phospholipids and membranes in a strictly calcium dependent manner (Burgoyne and Geisow, 1989; Raynal and Pollard, 1994). Members of the annexin family are built from the same mould: a core structure composed of 4 (32-39 kDa annexins) or 8 (annexin 6) homologous domains of conserved 70 amino-acid residues (Burgoyne and Geisow,1989; Raynal and Pollard, 1994).The N-terminal domain is nevertheless variable in length and sequence while remaining specific to each annexin (Drust and Creutz, 1988; Hoekstra *et al.*,1993) and it harbors phosphorylation sites in certain members (Moss *et al.*, 1991). Phosphorylation of the

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N-terminal domain plays an important role in the regulation of certain annexin functions (Porte *et al.*, 1996; Wang and Creutz, 1994).

Annexins have been well characterized at the structural and biochemical level. The three-dimensional structure of annexins 1 (Crégut *et al.*, 1994; Weng *et al.*, 1993), 2 (Burger *et al.*, 1996), 3 (Favier-Perron *et al.*, 1996), 4 (Zannoti *et al.*, 1998), 5 (Huber *et al.*, 1992), 6 (Benz *et al.*, 1996), 7 (Liemann *et al.*, 1997), 12 (Luecke *et al.*, 1995) and 24 (Hofmann *et al.*, 2000) has been determined. Their actual physiological functions are however far from understood. Various roles have been proposed for annexins. Importantly, they deal with processes involving membrane fusion and/or fission such as fusion of endosomes (Mayorga *et al.*, 1994), endocytosis (Lin *et al.*, 1992), phagocytosis (Diakonova *et al.*, 1997; Harricane *et al.*, 1996; Kusumawati *et al.*, 2000), exocytosis (Creutz, 1992; Sarafian *et al.*, 1991) or cellular traffficking (Creutz, 1992). Macrophages mainly contain annexin 1 but also annexin 2 (Isacke *et al.*, 1989) and annexin 3 (Le Cabec *et al.*, 1994). Annexin 1 has been reported to be associated with late stage phagosomes containing inert material (Diakonova *et al.*, 1997), yeast, non-pathogenic *E. coli* JM109, or dead intracellular pathogenic *Brucella* (Harricane *et al.*, 1996). This protein is however absent in late stage phagosomes containing live *Brucella* internalized with IgG opsonization (Harricane *et al.*, 1996) but interessingly it is a common constituent of the protein complex of early stage phagosomes regardless of whether they contain live pathogenic or non-pathogenic bacteria (Kusumawati *et al.*, 2000).

Green Fluorescent Protein (GFP) is one of the most popular reporter proteins. Owing to its autofluorescence, when fused to a protein of interest it can be directly used for the study of the cellular localization of that protein. In this study, we assessed the consequences of annexin 1 fusion to a reporter protein at its N-terminal end on functions related to phagocytosis.

Materials and Methods

Cell culture

The J-774A.1 murine macrophage-like cells (ATCC) were seeded the preceding day on glass coverslips at 1.10^5 cells/ml in 1 ml complete medium (RPMI 1640 supplemented with 5.10^{-3} M glutamine [Gibco] and 10% fetal calf serum [Sigma]) and grown in a 24-well culture plate at 37°C and 5% CO_2.

Phagocytosis of latex beads

Phagocytosis was carried out by internalizing latex beads (Sigma) (3 µm diameter ; 10% suspension diluted 1:200 in culture medium) into transfected J-774A.1 cells (see below). Cells grown on coverslips were exposed to latex beads for 5, 15 , or 30 min at 37°C, then extensively washed for 30 min then a further 1 hour incubation (post-infection step) after an extensive washing step, then again extensively washed.

Construction of eucaryotic expression plasmid

The cDNA of wild-type (WT) annexin 1 was inserted in the mammalian expression vector pEGFP-C1 (Clontech) and expressed as a fusioned protein to green fluorescent protein (GFP) at its N-terminal end. The cDNA of WT annexin I, cut from the previous construct pKA/WT (bacterial pKK 233-2-based expression vector) (Travé *et al.*, 1991), by *Nco*I then made blunt end by Klenow treatment, was first inserted in the *Sma*I site in pGFP-CI. The insert was then excized by *Xho*I and *Bam*HI and inserted in the same sites in pEGFP-C1. The resulting plasmid was called pEGC/AnWT. Plasmids

were purified using the Endofree plasmid kit (Qiagen) and introduced in J-774A.1 cells by transfection.

Transfection of J-774A.1 cells

Transfections were carried out using LipofectAmin™ (life Technologies). Briefly, 2 µg of plasmid (pEGC/AnWT, expressing annexin 1 fused to GFP; see Results) were first complexed to 10 µl of reagent for 15 min at room temperature then added to J-774A.1 cells (1. 10^6 cells in 1 ml of Opti-MEM (GIBCO-BRL), without serum, recommended for optimal transfection (Harrison *et al.*, 1995). Cells were incubated for 2 hours at 37°C then washed with RPMI 1640 and cultured in RPMI 1640-fetal calf serum for 24 hours at 37°C and 5% CO_2.

Immunocytochemistry

Following phagocytosis of latex beads, PBS-washed J-774A.1 cells were fixed with 3.7% formaldehyde in PBS for 15 min at room temperature or at 4°C overnight. They were permeabilized with 0.5% saponin in PBS. Annexin 1 was labeled with polyclonal anti-annexin 1 antibodies raised in rabbit (Harricane *et al.*, 1996), then revealed with rhodamine-labeled anti-rabbit antibodies raised in goat (Chemicon, Euromedex). Cells were mounted for fluorescence microscope observations.

Fluorescence microscopy observations

Fluorescence microscopy acquisitions were obtained with a Cool View camera (Photonic Science) and image processor linked to an inverted Leica DM IRB microscope (Leica). The filters used to detect GFP fluorescence consisted of an excitation bandpass filter (450 to 490 nm), a dichroic mirror (510 nm), and an emission bandpass filter (515 to 560 nm). The filters used to detect rhodamine fluorescence consisted of an excitation bandpass filter (515 to 560 nm), a dichroic mirror (580 nm), and a longpass emission filter (>590 nm).

Results and Discussion

Vector constructs expressing annexin 1 fused to GFP

Transient transfections are currently used to study the mechanism of function of proteins. We used this approach to investigate annexin 1 functions involved in phagocytosis. However, as the transfection efficiency of macrophagic cell lines J-774A.1 or U-937 cells is very low (Kusumawati *et al.*, 1999), and the expression level of the introduced cDNA is *a priori* not known, a reporter, i.e. GFP, was used to identify the transfected cells. Owing to the autofluorescence of GFP, fusion of annexin I to this reporter protein allows its direct visualization in living cells without addition of antibodies. In the first constructs, the cDNA of WT annexin 1 was inserted in the expression vectors pGFP-C1, harboring the initial sequence of *Aequorea victoria*, as described in the Materials and Methods. Positive clones were identified by *Eco*RV which only cut annexin 1 cDNA. Besides, as the insertion has been done in a blunt end site, two series of construct were obtained, one expressing mRNA in sense and one expressing mRNA in anti-sense. The orientation in sense or anti-sense was determined by *Eco*RI and *Hind*III (Fig. 1-A, Table 1).

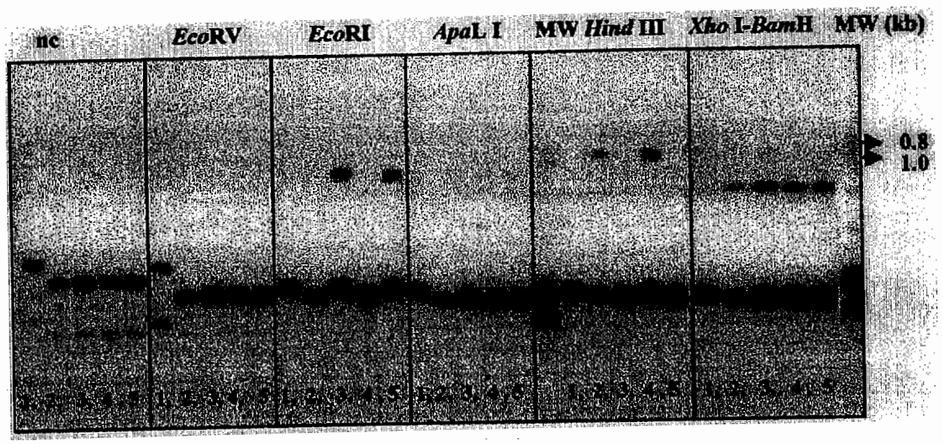


Fig. 1-A. Analyses of constructs based on pGFP-C1 (2,3) or on pEGFP-C1 (4,5) by Electrophoresis on agarose gel. Control (1), clones in sense orientation (2,4) and clones in anti-sense orientation (3,5).

Table 1. Digestion by restriction enzymes and prediction of the resulting DNA fragments. uc: un cut; lin: linnaraized

Restriction enzymes	pEGFP-C1	pEGC/An-sens	pEGC/An-anti-sens
*Eco*RV	uc	lin (6.761)	lin (6.751)
*Eco*RI	lin (4.731)	6.751; 50	5.721; 1.080
*Hind*II	lin	5.921; 870	6.531; 260

However, the GFP, contained in pGFP-C1, was poorly expressed in eukaryotic cells. Therefore we transferred annexin 1 cDNA in pEGFP-C1, harboring enhanced GFP, which is much more suitable for use in mammalian cells. As the two vectors contain the same multiple cloning sites, the insertion was easily done in oriented manner in *Xho*I/ *Bam*HI sites. The map of the resulting constructs, *i.e.* pEGC/ AnWT is given in Fig. 1-B. In pEGC/ AnWT (sense), annexin 1 is expressed under the control of a CMV promoter as a fusioned protein to GFP at its N-terminal end. The fusion was made by a peptide link of 19 amino-acids which has the following sequence:

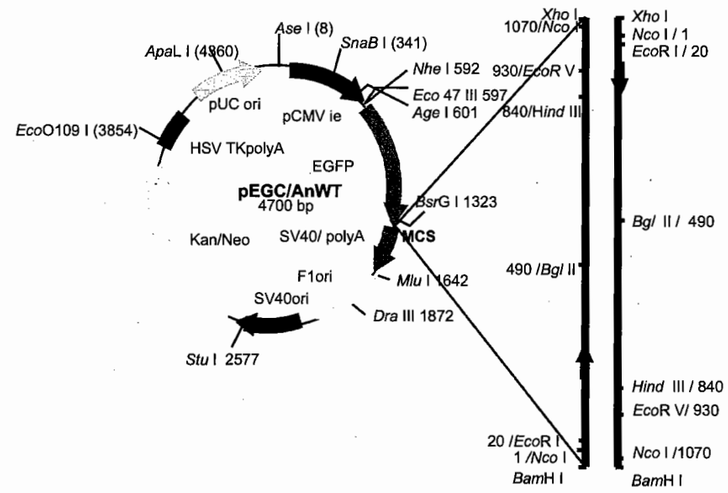
5' TCC GGA CTC AGA TCT CGA GCT CAA GCT TCG AAT TCT GCA
N- *S G L R S R A Q A S N S A*
GTC GAC GGT ACC GCG GGC 3'
V D G T A G -C

Cellular distribution and phagosome colocalization

Fusion of annexin 1 to GFP at its N-terminal end could disturb its properties by steric hindrance, modification of structural flexibility or shielding of active sites. Annexin 1 was fused to GFP via a peptide link of 19 amino-acids with the aim of obtaining some degree of flexibility. Nevertheless the fusion effect was not predictable since the N-terminal domain harbors regulatory sites, such as phosphorylation sites (Moss *et al.*, 1991).

The exogeneous annexin 1 was introduced in J-774A.1 cells by transfecting these cells with pEGC/ AnWT (sense), expressing annexin 1 fused to GFP, as described in the Materials and Methods. Transfected cells were identified by the green fluorescence which detected annexin 1 fused to GFP hence the introduced protein. Annexin 1, both endogeneous and exogeneous, was also

detected by rhodamine-labeled anti-annexin 1 antibodies. The fluorescence microscope observations showed much higher expression of exogeneous annexin 1 as compared to expression of the endogeneous protein. The level of expression of the introduced protein was at least twenty-fold that of the endogeneous form (data not shown). Therefore the effects observed will be mainly due to the introduced protein. This is particularly important when mutant forms are introduced in the cells as the effects of the endogeneous wild type form will be easily overcome by the exogeneous ones.



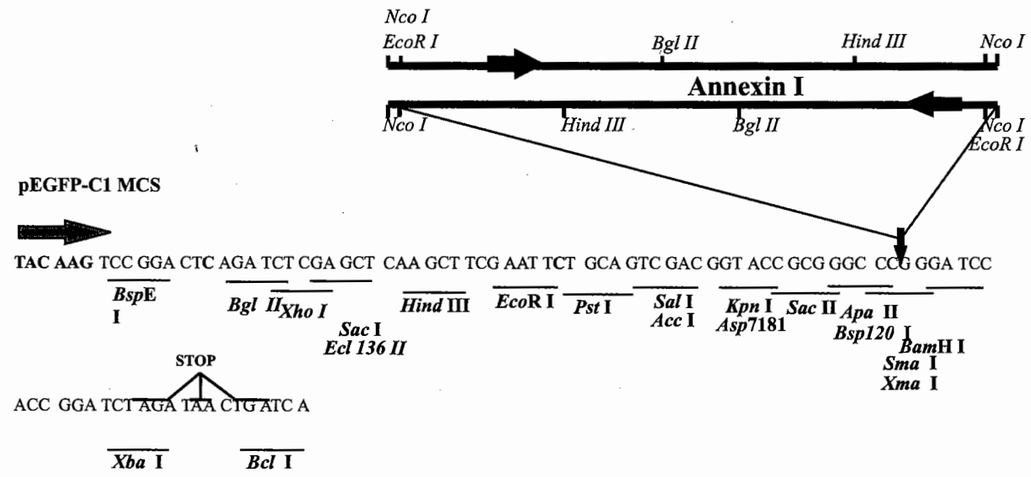


Fig. 1- B. Map of pEGC/ AnWT expressing annexin 1 fused to GFP, also showing the two orientations of annexin 1 cDNA with regard to the GFP gene and promoter.

The fusion of annexin 1 to GFP was used to investigate the implication of this protein in phagosome trafficking. Phagocytosis was carried out by internalizing latex beads into transfected J-774A.1 cells for various times as described in the Materials and Methods. The cellular distribution of annexin 1 is shown in Fig. 2. As seen, annexin 1 exhibited a granular pattern in the cytoplasm. Fused to GFP at its N-terminal end, annexin 1, detected by anti-annexin 1, was associated with phagosomes in granular structures, at early stage (Fig. 2-A,B), during the maturation process (Fig. 2-C) and at late stage (Fig. 2-D). Although already abundant in macrophages (Ernst *et al.*, 1996), its overexpression did not seem to affect the phagosome colocalization nor its association with granular structures. Annexin 1 can also be observed by its green fluorescence which identifies the exogeneous form. Nevertheless, when the green fluorescence was used, its granular pattern was less visible though the phagosome colocalization was still clearly observed (Fig. 2).

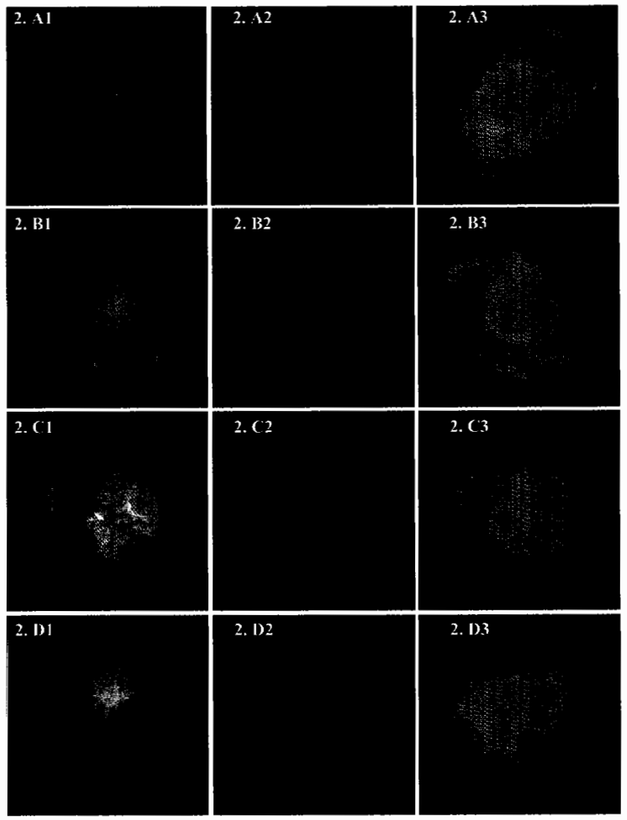


Fig. 2. Kinetic of phagocytosis of latex beads by pEGC/AnWT (sense)-transfected J-774A.1 Cells. Transfection was performed as described in the Materials and Methods.Phagocytosis was carried out for different times: 5 min (A), 15 min (B), 30 min (C) without post-infection incubation or 30 min followed by 1 h post-infection incubation (D). Annexin 1 was also detected with anti-annexin 1 antibodies then labeling with rhodamine-labeled secondary antibodies. Images were obtained under a fluorescence microscope: rhodamine and GFP labeling (1), rhodamine labeling alone (2) and GFP fluorescence alone (3)

We previously established that annexin 1 is found in early stage phagosomes regardless of whether they contain live pathogenic or non-pathogenic bacteria (Kusumawati *et al.*, 2000). This work shows that it is also a component of early phagosomes containing latex beads. It turns out so that annexin 1 is a common constituent of the protein complex of early stage phagosomes. Annexin 1 has been reported to be a constituent of late stage phagosomes containing inert material (Diakonova *et al.*, 1997; this work), non-pathogenic *E. coli* DH 5α, yeast or killed pathogenic *Brucella* (Harricane *et al.*, 1996; Kusumawati *et al.*, 2000). This may be correlated with the possible involvement of annexin 1 in membrane fusion and/or fission during phagosome maturation. Its absence in late stage phagosomes containing live *Brucella* (Harricane *et al.*, 1996; Kusumawati *et al.*, 2000) may constitute a mechanism exploited by this bacterium to survive inside phagosomes.

Despite extensive studies at the biochemical and structural level, the actual physiological functions of annexins have not yet been thoroughly understood. It is noteworthy that despite the regulatory importance of the N-terminal domain, the fusion of annexin 1 to GFP at its N-terminal end did not affect the functions associated with phagocytosis. The absence of any apparent inhibition of the annexin 1 functions considered is of great interest. Owing to the autofluorescence of GFP, GFP-tagged annexin 1 can so be directly visualized without labeling with antibodies. It allows therefore direct dynamic studies in living cells and in real time. This fusion approach can be extended to other proteins and be used for the study of mutant forms and naturally not occurring isoforms.

Acknowledgments

This study was supported by University Research for Graduate Education (URGE) IBRD Loan 3754-IND, the Ministry of Education and Culture for Indonesia, Inter University Center for Biotechnology, Gadjah Mada University, Indonesia, INSERM Institute National de la Sante´ et de la Recherche Médicale) and CROUS, French government.

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